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PRINCIPAL INVESTIGATOR: William M. Pardridge

PI ADDRESS: University of California, Los Angeles
405 Hilgard Avenue
Los Angeles, California 90024-1682

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William M. Pardridge, M.D.

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Most neuropeptides are incapable of entering brain from blood owing to the presence of unique anatomical structures in the brain capillary wall, which makes up the blood-brain barrier (BBB). Such neuropeptides could be introduced into the bloodstream by intranasal insufflation and, thus, could have powerful medicinal properties (e.g., β-endorphin for the treatment of pain, vasopressin analogues for treatment of memory, ACTH analogues for treatment of post-traumatic epilepsy), should these peptides be capable of traversing the BBB. One such strategy for peptide delivery through the BBB is the development of chimeric peptides, which is the basis of the present contract. The production of chimeric peptides involves the covalent coupling of a nontransportable peptide (e.g., β-endorphin, vasopressin) to a transportable vector peptide (e.g., insulin, transferrin, cationized albumin, histone). The transportable peptide is capable of penetrating the BBB via receptor-mediated or absorptive-mediated transcytosis. Therefore, the introduction of chimeric peptides allows the nontransportable peptide to traverse the BBB via a physiologic "piggy back" mechanism.

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William M. Packer 1/15/91

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A. INTRODUCTION

The developments in molecular neurobiology are allowing neurologic and psychiatric disorders to be defined in molecular terms (1). With the advent of this new knowledge will come the development of pharmaceutical compounds that are tailored to a specific disease on the basis of the molecular biology of that particular disorder. Invariably, such therapeutic compounds will be specific neuropeptides that are deficient in certain areas of the brain and the correction of this deficiency could allow for treatment of the neurologic or mental disorders. Possible disorders and the respective peptides that may be efficacious in the illness include: epilepsy (ACTH analogues), fever (MSH analogues), pain (β-endorphin), brain tumors (interleukins, monoclonal antibodies), depression (CRH antagonists), or cerebral AIDS (CD4 antigen) (1-7). In addition, neuropeptides may be used to optimize brain performance: memory (e.g., vasopressin analogues) (8).

Unfortunately, none of the above peptides are capable of traversing the brain capillary wall which makes up the blood-brain barrier (BBB) in vivo. The microvessels of all organs, except for the brain, are endowed with small pores that allow for small molecules such as neuropeptides to readily escape from blood into the respective organ (9). However, in the brain of all vertebrates these microvascular pores are completely absent and, thus, circulating neuropeptides do not gain access to brain interstitial space from the circulation (10). Since neuropeptides may be administered into the bloodstream conveniently by intranasal insufflation (11), the normal gut barriers to peptides may be easily traversed and the rate-limiting step in the development of peptides as neuropharmaceuticals is circumvention of the BBB.

There are three possible strategies for drug and neuropeptide delivery through the BBB (12): neurosurgical or invasive-based strategies, lipidization or pharmacologic-based strategies, and chimeric peptide or physiologic-based strategies. Of the neurosurgical-based strategies, there are two possibilities. First, attempts have been made to transport drugs across the BBB after intracarotid injections of hypertonic solutions (13). However, this is a highly invasive procedure that has a 15-20% side effect of seizures in experimental animals, must be performed in an operating room under heavy sedation, and is obviously not amenable to everyday use. The second type of neurosurgical or invasive strategy is the implantation of a chronic intraventricular catheter (14). However, this approach is limited by the fact that the pharmaceutical agent is only delivered to the surface of the brain with this approach. Owing to the fact that cerebrospinal fluid is pumped out of the brain faster than molecules can diffuse down into brain parenchyma, the delivery of neuropeptides into brain CSF by the catheter route would not allow for treatment of neurologic diseases that are not meningeal-based in etiology. The lipidization strategy involves converting water soluble drugs into lipid soluble pro-drugs. However, this approach cannot be used for neuropeptides. Recent studies form the Principal Investigator's laboratory have shown that cyclosporin, which is an extremely lipid soluble peptide, is not capable of crossing the BBB owing to it's molecular size of 1,100 Daltons (15). Therefore, even if the highly hydrophilic peptides were made lipid soluble, their large molecular weight would preclude

their transport through the BBB. Thus, the only apparent strategy known today for delivery of neuropeptides thorugh the BBB is the development of chimeric peptides.

The developent of chimeric peptides involves the coupling of a nontransportable peptides (e.g., β-endorphin) to a transportable peptide (e.g., insluin, tranferrin, cationized albumin, or histone). The transportable peptide vector is a molecule that is known to be transported through the BBB via either receptor-mediated (e.g., insulin, transferrin) or absorptive-mediated (e.g., cationized albumin, histone) transcytosis through the endothelial cytoplasm. First generation chimeric peptides involves covalent coupling of a nontransportable and transportable peptide using disulfide-based cross-linking reagents such as SPDP. Second generation chimeric peptides will undoubtedly involve recombinant DNA technology wherein genes of the transportable and nontransportable peptides are fused and the entire recombinant chimeric peptide is produced in large quantities in either prokaryotic or eukaryotic expression systems.

The aims of the present contract are four-fold: to synthesize chimeric peptides, to demonstrate their transport into brain interstitium by physiologic-based methods, to demonstrate morphologically the subcellular endothelial organelles involved in chimeric peptide trafficking, and to purify these vesicles with biochemical techniques.

The principal methodology for synthesis of the chimeric peptides was a chemical covalent coupling approach using N-succinimidyl-3-[2-pyridyldithio(proprionate)] (SPDP) (16). This reagent allows for disulfide-based coupling of the non-transportable peptide to the transport vector. This linker was chosen since disulfide bonds are generally stable in the bloodstream, but labile in tissues. With regards to the second specific aim directed at physiological experiments showing net transcytosis of chimeric peptides through the BBB, both in vivo and in vitro methodologies were employed. In vitro methods included the use of isolated animal and human brain capillaries as an in vitro model system of BBB receptor binding and endocytosis. However, transcytosis cannot be studied with isolated brain capillaries. Therefore, a second in vitro methodology was established, which employed the measurement of transcytosis of peptides through a monolayer of bovine brain capillary endothelial cells grown on polycarbonate filters and placed in side-by-side temperature controlled well-mixed transport chambers that allowed for the measurement of transport through the endothelial monolayer from the donor chamber to the acceptor chamber. The in vivo methodologies employed were an internal carotid artery in situ perfusion method and a single intravenous injection/pharmacokinetic analysis approach. Both methods utilized a capillary depletion technique to differentiate binding/endocytosis at the brain microvasculature from actual transcytosis of peptide into brain interstitial fluid. Morphologic identification of transport subcellular organelles employed both immunogold electron microscopy and autoradiography electron microscopy techniques. Isolation of subcellular organelles identified by the morphologic methods were to be carried out using Percoll gradient sedimentation techniques. However, the vesicle isolation methods were not undertaken, as insufficient progress in subcellular organelle identification using the morphologic techniques (i.e., specific aim #3) was achieved within the curtailed contract period.

B. BODY

The first priority was to identify the optimal model BBB transport vector. Experiments were performed to investigate cationized bovine albumin (17), human insulin growth factors (IGF) (18), cationized immunoglobulin G (IgG) (19), histone (20), acetylated low density lipoproteins (LDL) (21), and cationized rat albumin (22). With the exception of acetylated LDL, all of these vectors were initially examined with the isolated brain capillary radioreceptor assay. In addition, cationized bovine albumin was evaluated with the in vitro tissue culture transcytosis method. The transcytosis of cationized bovine albumin, cationized IgG, and histone were investigated with both the internal carotid artery perfusion technique and the intravenous injection/pharmacokinetic approach (17, 19, 20). Cationized rat albumin was evaluated with the intravenous/pharmacokinetic method (22). A number of factors must be considered in evaluating the best vector to be used in subsequent chimeric peptide studies. These factors are: (a) the rate of clearance of the vector from the bloodstream following administration by high uptake organs such as liver or kidney, (b) the potential toxicity of the vector, e.g., some cationized proteins cause glomerular nephropathies, (c) the potential antigenicity of the vector, i.e., some cationized heterologous proteins are highly immunogenic, (d) whether the vector undergoes true transcytosis into brain interstitial fluid as opposed to mere binding and endocytosis into brain capillary endothelial cytoplasm, e.g., acetylated LDL only undergoes endocytosis at the BBB and does not undergo transcytosis, and (e) the possible avid binding of the vector by serum proteins, e.g., the IGF is avidly bound by serum binding proteins. Cationized bovine albumin or IgG bind to serum proteins if the vector is radiolabeled with oxidative iodination as opposed to reductive tritiation (which causes no inhibitory binding with serum proteins). The use of cationized rat albumin in rats was found to fulfill all of the criteria for a promising vector, i.e., it was found not to be rapidly cleared by liver and to have a relatively long serum half-life in the order of several hours, was only weakly antigenic when administered repetitively to rats, exhibited no toxicity on peripheral organs following a daily administration for an 8-week period, underwent true transcytosis into brain interstitial fluid, and was not bound by serum proteins (22).

The model chimeric peptide employed was a β-endorphin cationized albumin chimeric peptide coupled with (SPDP) (23). In reference 23, the four principal components of the chimeric peptide scheme are discussed and are represented in Figure 1. The first step is the receptor-mediated or absorptive-mediated endocytosis of the chimeric peptide at the brain capillary endothelial wall. This step was studied with isolated brain capillaries using Scatchard analysis and a radioreceptor assay and acid wash assays as described in reference 17. The second step is receptor-mediated or absorptive-mediated exocytosis of the chimeric peptide at the brain capillary wall resulting in release of the chimeric peptide into brain interstitial fluid. This was documented in reference 23, using an internal carotid artery in situ perfusion technique coupled with a capillary depletion method. The third step is cleavage of the disulfide bond by cerebral disulfide reductases. This step was documented as described in reference 23, employing fast protein liquid

chromatography (FPLC) gel filtration. The fourth step in the overall chimeric peptide scheme is reactivity of a pharmacologically-active chimeric peptide with its respective brain receptor. In the case of the β-endorphin chimeric peptide prepared and studied in reference 23, pharmacologic studies cannot be performed since the N-terminal amino group was involved in the chimeric peptide synthesis. It is known that a free N-terminal amino group must be available for active binding to opioid receptors (23).

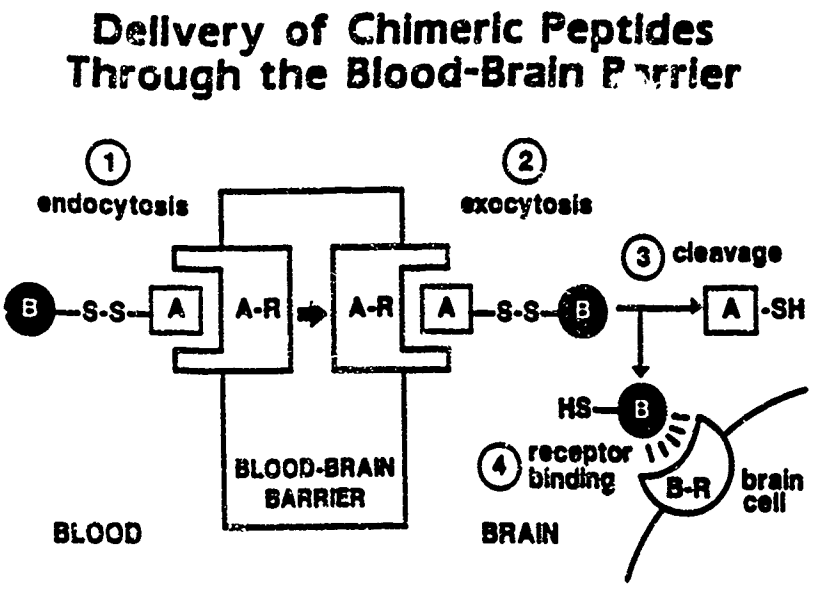

Figure 1

Having confirmed the operation of the first three steps in the overall chimeric scheme (Figure 1), the work in the final stages of the contract was aimed at determining the important structural features of the pharmacologically active peptide that must be present in order to preserve biologic activity following cleavage of the disulfide bond that results in release of free pharmaceutical peptide into brain interstitial fluid. These structural features that must be incorporated into the pharmacologically active peptide at the level of solid state peptide synthesis are as follows:

(a) The peptide must be relatively resistant to rapid amino-peptidase inactivation. This requires a substitution of glycine at the number 2 position with a D-alanine in the case of opioid peptides. Therefore, in reference 23, a β-endorphin analog, [D-Ala^2]β-endorphin (DABE) was studied.

(b) The N-terminal α-amino group must be protected during the coupling procedure that converts free amino groups to pyridyldithiolproprionate (PDP) moieties using disulfide coupling based reagents such as SPDP. This problem may be solved by preparing N-terminal t-butoxycarbonyl (BOC) derivatives of the opioid peptide by aminolytic cleavage of the peptide from the solid phase resin using 1,5-diaminopentane as described by Goldstein and collaborators (24).

(c) The opioid peptide must be metabolically active following its release from the drug transport vector after cleavage of the disulfide bond. This requirement necessitates that the "molecular baggage" that is left on the opioid amino group (see 23) following it's cleavage from the chimeric peptide is placed in the portion of the molecule that is not involved in biologic activity and receptor binding. In the case of dynorphin derivatives, this problem may be solved by replacing lysine groups with arginines and preparing an opioid analog with a

C-terminal extended amino group that may participate in the coupling reaction, but that is not important for receptor binding site activity (24). The importance of these structural features that must be incorporated into the pharmaceutical peptide at the level of solid state synthesis has recently been reviewed (25).

With respect to identification of subcellular organelles involved in the transcytosis process, this is the most difficult component of the contract work. Initially, electron microscopy immunogold procedures were performed. However, this required the availability of an antibody to either the vector compound or the pharmaceutical peptide. Therefore, we initially prepared an antiserum to cationize IgG. This antiserum was prepared in our laboratory, and was found to be highly specific for cationized IgG and did not react with native IgG molecules. However, the antiserum did react with endogenous ligands in brain tissue and this non-specific background was found to prohibit further studies. Therefore, we set up procedures for electron microscopic autoradiography. These experiments were initially successful after using high concentrations of radioactivity. However, the resolution of this technique was poor, and in the final stages of the contract, we are unable as yet to identify subcellular organelles involved in transcytosis. These autoradiography studies, however, did confirm earlier autoradiography studies that transcytosis into brain interstitium of these vectors does in fact take place (17, 19).

C. CONCLUSIONS

The work of this three-year contract solidified the experimental foundations for the chimeric peptide model for transport of pharmacologically active peptides through the BBB. With respect to future studies in humans, cationized human albumin should be examined as a BBB transport vector based on the promising studies using cationized rat albumin in rats (22). Cationized human albumin may be initially evaluated using isolated human brain capillaries and subsequently may be evaluated for toxicity in phase I clinical trials.

The final extension of the chimeric peptide model to step 4 (Figure 1) will involve the development of pharmacologic paradigms to demonstrate the in vivo efficacy of systemically administered chimeric neuropeptides. In this regard, it is important to identify peptide analogues that are predicted to be pharmacologically active following cleavage from the transport vector. During the final stages of the contract, a model 5 vasoactive intestinal peptide (VIP) analogue was identified (26), and this peptide can be used to measure increases in cerebral blood flow following systemic administration. Centrally-administered VIP increases cerebral blood flow, but systemically-administered VIP does not owing to lack of transport of VIP through the BBB. However, a model 5 VIP cationized rat albumin chimeric peptide may increase cerebral blood flow following systemic administration and this is experimentally testable. With regard to opioid peptides, the latter stages of the contract identified dynorphin analogues that would be suitable for preparation of biologically active chimeric peptides, and these are discussed in reference 25. The biologic activity of the opioid chimeric peptide

may be assessed in vivo in rats using the tail-flick analgesia paradigm. The use of cationized rat albumin has no toxic sequelae following repetitive administration (22), and the use of this vector will allow for repetitive pharmacologic testing in laboratory rats. With regard to subcellular identification of vesicles involved in the transcytosis process, it is recommended that 1 nm gold conjugates of cationized rat albumin be employed since this would eliminate the need for the primary antibody, which has a high non-specific binding, and a second gold-labeled antibody. Moreover, the use of 1 nm labeled vectors will be advantageous since this gold particle is much smaller than the standard gold particle (which is 5-20 nm) and the gold particle size will not impair the transport activities of the BBB transport vector. The size of the gold particle may be amplified at the electron microscopic level using silver enhancement techniques (27). In conclusion, the chimeric peptide model for delivery of pharmacologically active peptides through the BBB continues to be a promising approach, and there is considerable amount of work yet to be done. The returns on such work are potentially high since the development of BBB delivery strategies may create a new generation of pharmacologically active but safe neuropharmaceuticals.

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F. PAID CONTRACT PERSONNEL

Rena Bains
Gregory Banks
Ruben J. Boado, Ph.D.
Jody Buciak, B.S.
Vivek Dixit, Ph.D.
Catherine Farrell, Ph.D.
Christine Farrell, M.Sc.
Kristine Moon
Kenneth Mukai
Camille Nichols
Thanh Nguyen
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Kimi Tamura
Domingo Triguero, Ph.D.
Chris Tsuneishi
Jing Yang, M.S.
Ted Yu